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(54) Title: TRANSGENIC CROPS ACCUMULATING FRUCTOSE POLYMERS AND METHODS FOR THEIR PRODUCTION			
(57) Abstract A method for producing fructose polymers of various lengths through expression of plant-derived FTF genes in transgenic monocot plants is disclosed. Also disclosed are transgenic monocot plants and seeds derived from said plants wherein the level of fructan that accumulates in the cells of the transgenic monocot plants and seeds is increased when compared to the level of fructan that accumulates in the cells of monocot plants and that do not contain the instant chimeric gene(s) encoding plant-derived FTF genes.			

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TITLETRANSGENIC CROPS ACCUMULATING
FRUCTOSE POLYMERS AND METHODS FOR THEIR PRODUCTION

This application claims the benefit of U.S. Provisional Application

5 No. 60/077,727, filed March 12, 1998.

FIELD OF THE INVENTION

The present invention concerns methods for synthesis and accumulation of fructose polymers in transgenic maize (*Zea mays* L.) by selective expression of plant-derived fructosyltransferase genes.

10 TECHNICAL BACKGROUND

Higher plants accumulate various commercially useful carbohydrate polymers such as cellulose, starch and fructan. Starch and cellulose are currently used in numerous food and non-food applications in their native form, but are more likely to be enzymatically or chemically modified, which greatly expands 15 their usefulness.

Fructans are linear or branched polymers of repeating fructose residues. The number of residues contained in an individual polymer, also known as the degree of polymerization (DP), varies greatly depending on the source from which it is isolated. For example, fructan synthesized by fungal species, such as in 20 *Aspergillus syndowi* may contain only two or three fructose residues. By contrast, polymers with a DP of 5000 or greater are synthesized by several bacterial lines, including *Bacillus amyloliquefaciens* and *Streptococcus mutans*. Intermediate sized fructan, with a DP of 3 to 60, are found in over 40,000 plant species (*Science and Technology of Fructans*, (1993) M. Suzuki and N. Chatterton, eds. CRC Press 25 Inc., Boca Raton, FL, pp. 169-190).

Regardless of size, fructose polymers are not metabolized by humans. Because of this, and due to their relative sweetness, small fructans with a DP of 3-4 are used in a wide variety of low calorie food products. Polymer size is critical to its commercial use. High DP polymers are not sweet, however, they do 30 provide texture to food products very similar to that of fat. High DP fructan used as a fat replacer also contributes very little to the caloric value of the product.

Fructans are also considered to be an excellent source of fructose for the production of high fructose syrup (Fuchs, A. (1993) in *Science and Technology of Fructans*, M. Suzuki and N. Chatterton, eds. CRC Press Inc., Boca Raton, FL, 35 pp. 319-352). Simple hydrolysis of fructan into individual fructose residues has a tremendous advantage over the current, technically demanding process of enzymatically converting starch into high fructose syrup. Using fructan as the starting material would, therefore, significantly reduce production costs.

The commercial potential for fructan is extremely high, however, its use is severely limited due mainly to the high cost of production. Fructan used in low-calorie foods is currently produced by fermentation culture. Larger polymers synthesized by bacteria are not currently produced on a commercial scale.

5 Isolation from plants would reduce the production costs, but fructan is not found in many crops of agricultural importance. Traditional crops, adapted to wide growing regions, such as oat, wheat and barley accumulate fructan, but only at extremely low levels. Fructan is currently harvested from plants on a relatively small commercial scale and only from a single plant species, *Cichorium intybus*.

10 Transgenic crops accumulating fructan through expression of chimeric fructosyltransferase (FTF) genes would have a significant advantage over native fructan-storing plants by making use of established breeding programs, pest resistance and adaptation to a variety of growing regions throughout the world.

15 Examples of fructan synthesis in transgenic plants containing genes from bacterial species, such as *Bacillus*, *Streptococcus* and *Erwinia* have been reported (Caimi et al., (1996) *Plant Physiol.* 110:355-363; Ebskamp et al., (1994) *Biotechnol.* 12:272-275; Rober et al., (1996) *Planta* 199:528-536). Synthesis of fructan in these non-fructan-storing plants was demonstrated, but accumulation was often very low and in tissues where high levels of fructan were reported to have a

20 detrimental effect on plant development.

Several important differences between transgenic plants expressing chimeric bacterial FTF genes and native fructan-storing plants were reported. The most obvious difference was in the size of the polymers synthesized. Transgenic lines containing bacterial FTF genes accumulate fructan with a DP of greater than 25 5000 (Ebskamp et al., (1994) *Biotechnol.* 12:272-275; Caimi et al., (1996) *Plant Physiol.* 110:355-363). Polymers synthesized in transgenic plants are, therefore, several thousand times larger than fructans which accumulate in plants such as chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.).

30 Differences in the specificity for donor and acceptor molecules have also been reported for bacterial and plant FTFs. The bacterial enzymes are known to release significant amounts of fructose to water as an acceptor (invertase activity); whereas the plant enzymes do not have invertase activity (Chambert, R. and Petit-Glatron, M. (1993) in *Inulin and Inulin Containing Crops*, A. Fuchs ed. Elsevier Press, Amsterdam. pp. 259-266). Fructose, liberated from sucrose by invertase activity, can not be used to increase the length of a polymer. Bacterial FTFs, therefore, convert sucrose to fructan less efficiently than do the plant enzymes.

The two classes of FTFs also differ in their affinity for sucrose, the sole substrate. Jerusalem artichoke sucrose-sucrose-fructosyltransferase (SST) has a Km for sucrose reported to be approximately 100 mM (Koops, A. and Jonker, H., (1994) *J. Exp. Bot.* 45:1623-1631). By contrast, the bacterial enzyme has a much 5 lower Km of approximately 20 mM (Chambert, R., and Petit-Glatron, M. (1991) *Biochem. J.* 279:35-41). This difference may have a critical effect on fructan synthesis, resulting in higher or lower levels of accumulation, depending on the concentration of sucrose in the cell. The fundamental differences between FTF 10 enzymes prevents meaningful predictions regarding the outcome of expression of plant genes in transgenic tissue, based on expression of bacterial FTF genes.

Predicting whether or not fructan would accumulate in a transgenic line containing the plant-derived FTF genes could be significantly enhanced if a greater understanding of the fructan metabolic pathway in native fructan-storing plants existed. The currently accepted model for fructan synthesis in plants 15 suggests that synthesis is a two step reaction. The initial reaction involves the enzyme sucrose-sucrose-fructosyltransferase (SST). SST catalyzes the synthesis of a trisaccharide from two sucrose residues. The second step, chain elongation, is carried out by the enzyme fructan-fructan-fructosyltransferase (FFT), (Edelman J., and Jefford T. (1968) *New Phytol.* 67:517-531. The model has been applied to all 20 fructan-storing plants (ca 45000 species). However, it is based largely on data from a single species, *Helianthus tuberosus*, and has undergone several revisions. A recent study demonstrates that the SST can act alone in producing long chain 25 fructan (Van der Ende, W. and Van Laere, A., (1996) *J. Exp. Bot.* 47:1797-1803). Thus, additional revisions in the model are necessary and suggests that there is only a rudimentary knowledge of fructan synthesis in plants.

Examples of fructan synthesis in transgenic plants containing microbial or plant-derived FTF genes has been reported (Vijn, et al., (1997) *The Plant J.* 11:387-398; Smeekens et al., WO 96/01904; Van Tunen et al., WO 96/21023, Sevenier et al., (1998) *Nature Biotechnology* 16:843-846). This previous work 30 involves expression of microbial or plant-derived SST genes only in transgenic dicotyledenous (dicots) plants. The present invention describes a method of increasing the level of fructan synthesis in transgenic monocotyledonous plants containing plant-derived SST genes or plant-derived SST and FFT genes.

Numerous differences between monocotyledonous (monocots) plants and 35 dicots exist which inhibit useful extrapolation of events occurring in one plant based on data from another. These differences include, but are not limited to, the competition for sucrose as an energy source among biosynthetic pathways in various plant organs and among biosynthetic pathways in different plant species.

Dicots and monocots are known to differ significantly in the transport and metabolism of carbohydrate. For example, pea (*Pisum sativum* L.), a dicot, transports glucose-6-phosphate into amyloplasts, the site where starch synthesized and stored. In monocots, such as maize, ADPglucose is transported into the amyloplast (Denyer et al., (1996) *Plant Phys.* 112:779-785). This seemingly simple difference illustrates a profound difference in the metabolic pathways necessary for processing the various forms of carbohydrate transported into the amyloplast in the two separate plants.

5 Transport of sucrose in plants also differs among plant species.

10 10 Specialized cells (basal endosperm transfer cells or BET cells) are adapted for the transport and metabolism of sucrose in maize kernels. The majority (greater than 90%) of sucrose transported to maize seeds is believed to be hydrolyzed in the specialized BET layer (Shannon, J. (1972) *Plant Physiol.* 49:198-202). The resulting hexose sugars are transported to the developing endosperm cells and

15 15 resynthesized as sucrose prior to entering the starch biosynthetic pathway. In contrast to maize, sucrose is directly transported to tubers of potato plants and enters the starch pathway unhydrolyzed (Opalka, K. and Wright, K. (1988) *Planta* 174:123-126).

20 Although poorly understood, exploiting the differences between monocots and dicots could not be considered a new concept. These differences are what drives the commercialization of herbicides such as 2,4-D which is tremendously toxic to dicots, but has no effect on monocot species. In this light, it seems clear that recent examples of transgenic dicot species containing a plant derived FTF gene (Vijn, et al., (1997) *The Plant J.* 11:387-398; Smeekens et al., WO 96/01904; 25 Van Tunen et al., WO 96/21023; Sevenier et al., (1998) *Nature Biotechnology* 16:843-846) can have no bearing on predicting the successful expression of FTF genes in monocot species. Variations in carbohydrate concentration, transport and metabolism among plant species, especially between monocots and dicots, are clearly too great to allow generalization.

30 SUMMARY OF THE INVENTION

35 This invention discloses a method for producing fructose polymers of various lengths through expression of plant-derived FTF genes in a transgenic monocot species. More specifically, the invention describes a chimeric gene comprising a tissue specific promoter, operably linked to the coding sequence for a sucrose-sucrose-fructosyltransferase gene (SST; EC 2.4.1.99) such that said chimeric gene is capable of transforming a monocot plant cell resulting in production of fructan with no deleterious effect on the said plant cell.

The invention further describes a chimeric gene comprising a tissue specific promoter, operably linked to the coding sequence for a fructan-fructan-fructosyltransferase gene (FFT; EC 2.4.1.100) such that said chimeric gene is capable of transforming a transformed plant cell (harboring a chimeric gene 5 comprising a tissue specific promoter, operably linked to the coding sequence for a sucrose-sucrose-fructosyltransferase gene (SST; EC 2.4.1.99)) resulting in production of fructan, with no deleterious effect on the said plant cell.

The invention also includes a monocot plant transformed with one or both of the chimeric genes described above, such that the plant produces fructan. The 10 invention also concerns a method of producing fructose or fructose polymers comprising growing the plant, harvesting the plant, and extracting fructan from the harvested plant.

The invention further describes a chimeric gene comprising a tissue specific promoter, operably linked to the coding sequence for a sucrose-sucrose-fructosyltransferase gene (SST; EC 2.4.1.99) such that the chimeric gene is 15 capable of transforming a monocot plant cell resulting in production of fructose polymers containing 2 to 3 fructose residues, with no deleterious effect on the transformed plant cell.

The present invention is not limited to naturally occurring fructosyl- 20 transferases but may equally well be performed by using modified versions thereof. Modifications may influence the activity of the fructosyltransferase in such a way that, for example, the degree of polymerization or the structure of the fructan produced is altered. Furthermore, according to the present invention a 25 single fructosyltransferase gene or a combination of fructosyltransferase genes of plant origin may be used.

The induced accumulation of fructans in transgenic plants using the 30 principles described herein will allow for the extraction of fructans from these plants for the purpose of fructan production. Fructans can accumulate in these plants (e.g., in harvestable organs such as roots, leaves, stems and seeds). Furthermore, the present invention further relates to seeds, cuttings or other parts 35 of the transgenic plants which are useful for the continuous production of further generations of said plants.

The fructans produced using transgenic plants of the present invention may be used in various food and non-food applications. Examples include but are not 35 limited to human and animal food products, in the production of fructose syrups, in the production of chemicals and plastics either as such or in a modified form.

Genetically modified crop plants which incorporate the fructosyl-transferase-encoding constructs mentioned above will allow for the efficient production of high quality carbohydrate polymers useful to man.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows a diagram of the cassettes used to express the Jerusalem artichoke SST and FFT genes (10 kD-SST and 10 kD-FFT, respectively) in transgenic maize endosperm. Each construct also contains the tissue specific 10 kD zein promoter and 3' transcription termination region.

10 Figure 2 shows Southern Blot analysis of leaf tissue from fourteen independently transformed lines containing only the 10 kD-SST cassette. Genomic DNA was digested with the restriction enzyme Bgl II. The complete 2.0 Kb coding sequence of the SST gene was labeled with ^{32}P and used to probe genomic DNA that was previously transferred to nylon membranes. Multiple intact (indicated by the arrow) and rearranged copies of the SST gene were shown 15 to be present in several of the transgenic maize lines.

20 Figure 3 shows TLC analysis of individual seeds from three transgenic lines containing intact copies of the 10 kD-SST expression cassette. Fructose, F; Sucrose, S; and fructan polymers containing 1 or 2 additional fructose residues (DP3 and DP4, respectively) are indicated. A marker lane (M) containing fructose, sucrose, DP3 and DP4 fructans is also indicated.

25 Figure 4 shows Southern Blot analysis of leaf tissue from twenty independent transformed lines containing the 10 kD-SST and 10 kD-FFT cassettes. DNA from lines previously shown to contain at least one intact copy of the 10 kD-SST cassette were digested with the restriction enzymes Eco RI and Bam HI. The complete coding sequence of the FFT gene was labeled with ^{32}P and used to re-probe genomic DNA. Multiple intact (indicated by the arrow) and rearranged copies of the FFT gene were shown to be present in several of the transformed lines.

30 Figure 5 shows TLC analysis of individual seeds from two transgenic lines containing intact copies of both the 10 kD-SST and 10 kD-FFT expression cassettes. Fructose polymers, larger than DP3 were demonstrated in seeds from each of the two lines. Fructose, F; Sucrose, S; and fructan polymers containing 1 and 2 additional fructose residues (DP3 and DP4, respectively) are indicated. A marker lane (M) containing fructose, sucrose, DP3 and DP4 fructans is indicated.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

In the context of this disclosure, a number of terms shall be utilized. As used herein, the term "nucleic acid" refers to a large molecule which can be

single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of the information in DNA into proteins. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

As used herein, "substantially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which results in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another hydrophobic amino acid residue such as glycine, valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that "substantially similar" sequences encompassed by this invention can also be defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the sequences exemplified herein.

“Gene” refers to a nucleic acid fragment that encodes all or a portion of a specific protein, and includes regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. “Native gene” refers to the gene as found in nature with its own regulatory sequences. “Chimeric gene” refers to a 5 gene comprising heterogeneous regulatory and coding sequences. “Endogenous gene” refers to the native gene normally found in its natural location in the genome. A “foreign gene” refers to a gene not normally found in the host organism but that is introduced by gene transfer. “Foreign gene” can also refer to a gene that is normally found in the host organism, but that is reintroduced at a 10 location in the genome where it is not normally found, resulting in one or more additional copies of the coding sequence of an endogenous gene.

“Coding sequence” refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

“Initiation codon” and “termination codon” refer to a unit of three adjacent 15 nucleotides in a coding sequence that specifies initiation and chain termination, respectively, of protein synthesis (mRNA translation). “Open reading frame” refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

“RNA transcript” refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect 20 complementary copy of the DNA sequence, it is referred to as the “primary transcript” or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript. “Messenger RNA” (mRNA) refers to RNA that can be translated into protein by the cell. “cDNA” refers to a double-stranded 25 DNA, one strand of which is complementary to and derived from mRNA by reverse transcription.

As used herein, suitable “regulatory sequences” refer to nucleotide 30 sequences located upstream (5'), within, and/or downstream (3') to a coding sequence, which control the transcription and/or expression of the coding sequences. These regulatory sequences include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences. In artificial DNA constructs, regulatory sequences can also control the transcription and stability of antisense RNA.

“Promoter” refers to a DNA sequence in a gene, usually upstream (5') to its 35 coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of

transcription initiation in response to physiological or developmental conditions. It may also contain enhancer elements.

An "enhancer" is a DNA sequence that can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to 5 enhance the level and/or tissue-specificity of a promoter. "Constitutive" promoters refer to those that direct gene expression in substantially all tissues and demonstrate little temporal or developmental regulation. "Tissue-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific tissues, such as leaves or seeds, or at 10 specific developmental stages in a tissue, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene 15 (i.e., a gene encoding a fructosyltransferase) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of a functional end-product encoded by a gene. More particularly, "expression" 20 refers to the transcription of the sense (mRNA) or the antisense RNA derived from the nucleic acid fragment(s) of the invention that, in conjunction with the protein apparatus of the cell, results in altered levels of protein product. "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

25 The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

30 "Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms.

35 "Fructosyltransferase" refers to a protein coded for by any one of several plant genes having the property of producing a carbohydrate polymer consisting of repeating fructose residues. The repeating fructose residues may be linked by α 2-1 linkage or a α 2-6 linkage or any combination of the two linkage types. The polymer of repeating fructose residues may contain one terminal glucose residue,

derived from a sucrose molecule, and at least two fructose residues. The polymer of repeating fructose residues in any form, with any combination of linkages, and with any number of fructose residues, is referred to generally as a "fructan".

A "fructosyltransferase gene" or "FTF" refers to the DNA sequence coding for a fructosyltransferase protein. The term "deleterious effect" as used herein, refers to a direct or indirect injurious effect on a plant or plant cell as a result of fructan accumulation, such that the plant or plant cell is prevented from performing certain functions including, but not limited to, synthesis and transport of carbohydrates within a cell and throughout the plant, regeneration of transgenic plants or tissue, development of the plant or plant cell to maturity, or the ability to pass the desired trait or traits to progeny.

The present invention describes chimeric genes comprising tissue specific regulatory sequences, FTF coding sequences and a transcription terminating region. The chimeric gene is capable of mediating the synthesis of a fructose polymer using sucrose as a substrate when expressed in a transgenic monocot plant wherein expression of the FTF gene results in the synthesis of novel fructose polymers, useful in numerous food and industrial applications. A transgenic corn plant (*Zea mays*) properly expressing the FTF gene, distinguishes itself from a generic plant of the same species by the presence of fructan accumulation in the mature seeds.

Transfer of the nucleic acid fragments of this invention into a plant directs expression of the protein in a manner that results in accumulation of this useful polymer, without concern for loss or alteration of the polymer due to plant degradatory enzymes during harvest, transport, or storage and without the loss of established co-products from any particular species. Transgenic crops containing chimeric genes comprising tissue specific regulatory sequences, the FTF gene and a transcription termination region will provide a renewable source of small (DP 2-3) and large molecular weight fructose polymers. Accumulation of fructan will be determined in part, by the level of expression of the chimeric gene in transformed crops. The level of expression depends in part, on the tissue specific expression signals, the number of copies of the gene integrated into the plant genome and location of gene integration; fructan accumulation may also be subject to substrate availability. The amount of substrate available to the enzyme depends on the species (including mutants within a species), the tissue type where expression occurs, the subcellular location of expression and on the stage of development of a particular plant. The stability of the introduced protein may also influence fructan accumulation and depends in part, on its proper processing, intracellular targeting and its ability to function in a foreign environment.

Successful expression of a gene with carbohydrate metabolic properties such as the Jerusalem artichoke SST and FFT genes, in a transgenic plant would require consideration of the following factors: (1) the species transformed, (2) the specific tissue where expression is to occur, (3) and the timing of expression. All 5 of these factors must be carefully coordinated in order for production of fructan to occur in a transgenic cell, with no deleterious effect.

Expression of a gene with sucrose metabolizing activity, such as an FFT protein, in a specific transgenic plant species would not necessarily create the same, or even a desired effect when expressed in a different plant species.

10 Differences in carbohydrate profiles among species suggests that an enzyme specific for sucrose will not always have sufficient substrate available to produce the same result when expressed in various species. It is well established that the availability of sucrose as a substrate not only varies greatly from species to species but also in mutants within the same species, (Lampe et al. (1931) *Bot. Gaz.*, 91:337-380).

Mechanisms for sucrose transport and accumulation in tissue also vary greatly from one species to another. Sucrose hydrolysis is an integral part of the import mechanism in developing corn seed, (Porter et al., (1985) *Plant Phys.*, 77:524-531), but is not a prerequisite for transport to developing soybean embryo 20 (Thorne, (1982) *Plant Phys.*, 70:953-958), or to wheat endosperm (Jenner, *Aust. J. Plant Phys.*, 1:319-329 (1974)). Therefore, expression of a FFT in the seed of one species may have access to an abundance of sucrose, however, fructan synthesis in seed of another species could be severely hindered by the accumulation of hexoses sugars in place of sucrose.

25 Tissue and developmental specific expression of a gene may be intrinsic to the promoter, the 3' non-coding region or combinations of the two, used in chimeric constructs. Promoters utilized to drive gene expression in transgenic plants can be derived from many sources so long as the chosen promoter(s) have sufficient transcriptional activity to accomplish the invention by expressing 30 translatable mRNA in the desired host tissue. Preferred promoters are those that allow expression specifically in seeds. Examples of seed-specific promoters include, but are not limited to, the promoters of seed storage proteins. The seed storage proteins are strictly regulated, being expressed almost exclusively in seeds in a highly organ-specific and stage-specific manner (Higgins et al. (1984) *Ann. Rev. Plant Physiol.* 35:191-221; Goldberg et al. (1989) *Cell* 56:149-160; Thompson et al. (1989) *BioEssays* 10:108-113). Moreover, different seed storage proteins may be expressed at different stages of seed development.

There are currently numerous examples for seed-specific expression of seed storage protein genes in transgenic plants. These include genes from monocots such as for barley β -hordein (Marris et al. (1988) *Plant Mol. Biol.* 10:359-366) and wheat glutenin (Colot et al. (1987) *EMBO J.* 6:3559-3564).

5 Moreover, promoters of seed-specific genes, operably linked to heterologous coding sequences in chimeric gene constructs, also maintain their temporal and spatial expression pattern in transgenic plants. Such examples include linking either the Phaseolin or *Arabidopsis* 2S albumin promoters to the Brazil nut 2S albumin coding sequence and expressing such combinations in tobacco,

10 *Arabidopsis*, or *Brassica napus* (Altenbach et al. (1989) *Plant Mol. Biol.* 13:513-522; Altenbach et al. (1992) *Plant Mol. Biol.* 18:235-245; De Clercq et al. (1990) *Plant Physiol.* 94:970-979), bean lectin and bean β -phaseolin promoters to express luciferase (Riggs et al. (1989) *Plant Sci.* 63:47-57), and wheat glutenin promoters to express chloramphenicol acetyl transferase (Colot et al. (1987) *EMBO J.* 6:3559-3564).

15 Of particular use in the expression of the nucleic acid fragment(s) of the invention will be promoters from several extensively characterized corn seed storage protein genes such as endosperm-specific promoters from the 10 kD zein gene (Kirihara et al. (1988) *Gene* 71:359-370), the 15 kD zein gene (Hoffman et al. (1987) *EMBO J.* 6:3213-3221; Schernthaner et al. (1988) *EMBO J.* 7:1249-1253; Williamson et al. (1988) *Plant Physiol.* 88:1002-1007), the 27 kD zein gene (Prat et al. (1987) *Gene* 52:51-49; Gallardo et al. (1988) *Plant Sci.* 54:211-281), and the 19 kD zein gene (Marks et al. (1985) *J. Biol. Chem.* 260:16451-16459). The relative transcriptional activities of these promoters in corn have been reported (Kodrzyck et al. (1989) *Plant Cell* 1:105-114) providing a basis for choosing a promoter for use in chimeric gene constructs for corn.

20 Moreover, promoters that drive the expression of genes encoding enzymes involved in starch biosynthesis may be used in the practice of this invention. These include the 5' regulatory sequences of the sucrose synthase (Yang, N.-S. and

25 Russell, D. (1990) *Proc. Natl. Acad. Sci.* 87:4144-4148) and the waxy or granule-bound starch synthase I (Unger et al. (1991) *Plant Physiol.* 96:124) genes.

30 Promoter elements may be derived from other starch synthase (granule-bound and soluble isoforms) genes when these become available, and from the sh2 (Bhave et al. (1990) *Plant Cell* 2:581-588) and bt2 (Bae et al. (1990) *Maydica* 35:317-322) genes whose products constitute the enzyme ADP-glucose pyrophosphorylase. It

35 is envisioned that the introduction of enhancers or enhancer-like elements into other promoter constructs will also provide increased levels of primary transcription to accomplish the invention. These would include viral enhancers

such as that found in the 35S promoter (Odell et al. (1988) *Plant Mol. Biol.* 10:263-272), enhancers from the opine genes (Fromm et al. (1989) *Plant Cell* 1:977-984), or enhancers from any other source that result in increased transcription when placed into a promoter operably linked to the nucleic acid 5 fragment of the invention.

Introns isolated from the maize Adh-1 and Bz-1 genes (Callis et al. (1987) *Genes Dev.* 1:1183-1200), and intron 1 and exon 1 of the maize Shrunken-1 (sh-1) gene (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207) may also be of use to increase expression of introduced genes. Results with the first intron of the maize 10 alcohol dehydrogenase (Adh-1) gene indicate that when this DNA element is placed within the transcriptional unit of a heterologous gene, mRNA levels can be increased by 6.7-fold over normal levels. Similar levels of intron enhancement have been observed using intron 3 of a maize actin gene (Luehrs, K. R. and Walbot, V. (1991) *Mol. Gen. Genet.* 225:81-93). Enhancement of gene expression 15 by Adh1 intron 6 (Oard et al. (1989) *Plant Cell Rep.* 8:156-160) has also been noted. Exon 1 and intron 1 of the maize sh-1 gene have been shown to individually increase expression of reporter genes in maize suspension cultures by 10 and 100-fold, respectively. When used in combination, these elements have been shown to produce up to 1000-fold stimulation of reporter gene expression 20 (Maas et al. (1991) *Plant Mol. Biol.* 16:199-207).

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for proper expression can be used to accomplish the invention. This would include the 3' end from any storage protein such as the 3' end of the 10 kd, 15 kd, 27 kd and alpha zein genes, the 3' 25 end of the bean phaseolin gene, the 3' end of the soybean β -conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the 30 necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions (for example, see Ingelbrecht et al. (1989) *Plant Cell* 1:671-680).

35 A number of genes from plant sources encoding enzymes with FTF activity have been isolated and sequenced. These include the SST and FFT genes from onion (*Allium cepa* L.), barley (*Hordeum vulgare* L.) and Jerusalem artichoke (*Helianthus tuberosus*); (Vijn et al., (1997) *Plant J.* 11:387-398;

Sprenger et al., (1997) *Febs Lett.* 400:355-358; Van Tunen et al., WO 96/21023; Smeekens et al., WO 96/01904). Preferred among these are the plant-derived SST and FFT genes from Jerusalem artichoke.

The SST and FFT genes can be isolated by techniques routinely employed by the skilled artisan for isolation of genes when the nucleotide sequence of the desired gene is known, or when the sequence of a homologous gene from another organism is known. Sequence information about the desired gene can be used to prepare oligonucleotide probes for identification and isolation of the entire gene from an appropriate genetic library. This library may be a genomic library, 5 wherein the coding region may be contained on a single DNA fragment or may be contained on several distinct DNA fragments. Alternatively, the library may be a cDNA library wherein the likelihood of isolating a cDNA clone comprising the entire coding region as one contiguous sequence is greater. In either instance, the appropriate clone(s) can be identified by DNA-DNA hybridization with probes 10 corresponding to one or more portions of the desired genes. Alternatively, oligonucleotide primers can be prepared and employed as PCR primers in order to 15 amplify and subsequently isolate all or part of the coding region from genomic DNA, or from the genomic or cDNA libraries described above.

Several different assays can be used to detect expression of the chimeric SST genes in seeds of the transformed plants. RNA transcripts, specific to the FFT genes may be detected by Southern or northern analysis. The FFT protein can be extracted, detected and quantified immunologically by methods known to those skilled in the art. Alternatively seed tissue may be ground and extracted with a polar solution, isolating and concentrating polysaccharides, including fructans, 25 which can then be detected by: TLC analysis, combined with a kestose specific stain (Wise et al., (1955) *Anal. Chem.* 27:33-36); HPLC analysis using fructan standards (Chatterton et al. (1993) In: Fuchs A. ed. *Inulin and inulin-containing crops*. Elsevier, Amsterdam pp. 93-99); or hydrolysis followed and an enzymatic-linked assay (Brown, C. and Huber, S. (1987) *Physiol. Plant* 70:537-543).

Various methods of introducing a DNA sequence (chimeric constructs containing SST or SST/FFT genes) into eukaryotic cells (i.e., transformation) of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (see 30 Klein et al. (1987) *Nature* (London) 327:70-73, and see U.S. Pat. No. 4,945,050), as well as those based on transformation vectors based on the Ti and Ri plasmids 35 of *Agrobacterium spp.*, particularly the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous

and dicotyledonous plants, such as soybean, cotton and rape (Pacciotti et al. (1985) *Bio/Technology* 3:241; Byrne et al. (1987) *Plant Cell, Tissue and Organ Culture* 8:3; Sukhapinda et al. (1987) *Plant Mol. Biol.* 8:209-216; Lorz et al. (1985) *Mol. Gen. Genet.* 199:178-182; Potrykus et al. (1985) *Mol. Gen. Genet.* 199:183-188).

Other transformation methods for chimeric constructs containing SST or SST/FFT genes are available to those skilled in the art, such as direct uptake of foreign DNA constructs (see EPO publication 0 295 959 A2), and techniques of electroporation (see Fromm et al. (1986) *Nature* (London) 319:791-793). Once 10 transformed, the cells can be regenerated by those skilled in the art. Also relevant are several recently described methods of introducing nucleic acid fragments into commercially important crops, such as rapeseed (see De Block et al. (1989) *Plant Physiol.* 91:694-701), sunflower (Everett et al., (1987) *Bio/Technology* 5:1201-1204), soybean (McCabe et al. (1988) *Bio/Technology* 6:923-926; Hinchee 15 et al. (1988) *Bio/Technology* 6:915-922; Chee et al. (1989) *Plant Physiol.* 91:1212-1218; Christou et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7500-7504; EPO Publication 0 301 749 A2), and corn (Gordon-Kamm et al. (1990) *Plant Cell* 2:603-618; Fromm et al. (1990) *Bio/Technology* 8:833-839). One skilled in the art is familiar with still other means for the production of transgenic maize plants 20 including introduction of DNA into protoplasts and regeneration of plants from said protoplasts (Omirulleh et al. (1993) *Plant Mol. Biol.* 21:415-423), electroporation of intact tissues (D'Hulluin et al. (1992) *Plant Cell* 4:1495-1505; Laursen et al. (1994) *Plant Mol. Biol.* 24:51-61), silica carbide mediated fiber transformation of maize cells (Kaeppeler et al. (1992) *Theor. Appl. Genet.* 84:560-566; Frame et al. (1994) *Plant J.* 6:941-948). In addition to the method of 25 particle bombardment of maize callus cells described above, one skilled in the art is familiar with particle bombardment of maize scutellar or suspension cultures to yield fertile transgenic plants (Koziel et al. (1993) *Bio/Technology* 11:194-200; Walters et al. (1992) *Plant Mol. Biol.* 18:189-200).

Once transgenic plants are obtained by one of the methods described 30 above, it will be necessary to screen individual transgenics for those that most effectively display the desired phenotype. It is well known to those skilled in the art that individual transgenic plants carrying the same construct may differ in expression levels; this phenomenon is commonly referred to as "position effect". 35 For example, when the construct in question is designed to express higher levels of the gene of interest, individual plants will vary in the amount of the protein produced and thus in enzyme activity; this in turn will effect the phenotype. This should not be seen as a limitation on the present invention, but instead as practical

matter that is appreciated and anticipated by the person skilled in this art. Accordingly, skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds, and is not an inherent part of the invention.

5

EXAMPLES

The present invention is further defined in the following examples. It will be understood that the examples are given for illustration only and the present invention is not limited to uses described in the examples. The present invention can be used to generate transgenic corn plants whose seed carbohydrate profile is altered by accumulation of fructose polymers and where its properties are useful such as in, but not limited to, foods, paper, plastics, adhesives, or paint. From the above discussion and the following examples, one skilled in the art can ascertain, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

10 All such modifications are intended to fall within the scope of the intended claims.

15

EXAMPLE 1

Chimeric Construct for Expression of the Jerusalem Artichoke SST Gene in Transgenic *Zea mays* L.

A construct designed for tissue specific expression of the Jerusalem artichoke SST gene in maize endosperm was assembled by replacing the Cauliflower Mosaic Virus (CaMV) 35S promoter in the plasmid pSST403 (Van Tunen et al., WO 96/21023) with a maize endosperm-specific 10 kD zein, seed storage gene promoter (Kirihsara et al. (1988) *Gene* 71:359-370). The complete SST coding sequence contained in pSST403 (Van Tunen et al., WO 96/21023) was isolated by digesting with the restriction endonuclease enzymes NcoI and HindIII. The isolated sequence was added to the plasmid pCyt-SacB (Caimi et al. (1996) *Plant Physiol.* 110:355-363) containing a 10 kD zein promoter and 3' termination region. The maize endosperm expression cassette, containing the 10 Kd promoter, SST coding sequence (including the native secretory and vacuole targeting signals) and the 10 kD 3' end, designated 10 kD-SST (Figure 1), was isolated by digesting with SmaI and SalI, then ligated into the plasmid KS17. The KS17 vector contains a hygromycin resistance gene (HPT) used as the selectable marker. The final vector was designated 10 kD-SST-17 was used directly for transformation into corn by particle bombardment.

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Plant Material and Transformation

The plant expression vector 10 kD-SST-17 and a plasmid vector encoding a selectable marker (pDetric) were cotransformed into embryogenic corn callus derived from crosses of the inbred lines A188 and B73 (Armstrong et al. (1991)

5 *Maize Genetics Cooperation Newsletter* 65:92-93) by microprojectile bombardment (Klein et. al., (1987) *Nature* 327:70-73). Transformed embryogenic cells were recovered on medium containing either glufosinate-ammonium or chlorsulfuron. The selectable marker pDetric contains the BAR gene (Thompson et al. (1987) *The EMBO Journal* 6:2519-2523), coding for phosphinothricin acetyltransferase, under the control of the 35S promoter. pALSLUC (Fromm, et al, (1990) *Biotechnology* 8:833-839), a plasmid vector encoding a mutant acetolactate synthase (ALS) gene that confers resistance to chlorsulfuron could also be used as a selectable marker. Expression of the mutant ALS gene is
10 regulated by the CaMV 35S promoter. Transgenic shoots were transferred to 12 inch pots containing METROMIX™ (Scotts-Sierra company) soil and grown to maturity in the greenhouse. Mature R₁ seed from original transformants were grown in the greenhouse or planted directly in the field.

Analysis of Transgenic Plants Expressing the SST Gene

15 Detection of the SST gene in transgenic plants was accomplished by PCR analysis, using oligonucleotide primers specific for the SST gene:

SST-1: 5'-ATGAATCCTTCATCCACCACGACCACCCCTCTC-3' (SEQ ID NO:1)

SST-2: 5'-CCCAGGAAGAGGGAAAGGATTGAGTTCTGCTTCCCC-3' (SEQ ID NO:2)

20 Confirming the presence of the SST gene in transgenic tissue and estimating the copy number was done by Sounthern Blot analysis, using the complete 2.0 Kb SST coding sequence. Southern analysis demonstrated the presence of multiple intact and rearranged copies of the SST gene in transgenic lines (Figure 2).

Carbohydrate Analysis of Transgenic Maize Lines Containing the SST Gene

Individual seeds from transgenic lines were harvested at 25-35 days post-pollination (DPP) for detection of fructose polymers. The seeds were crushed with a mortar and pestle. A small amount of water (200-400 uL) was added and 30 the mixture heated to 80°C for 10 minutes. The homogenized tissue was centrifuged at 10,000 x g for 10 minutes and 2 uL of aqueous solution spotted on HP-K silica TLC plates (Whatman Scientific, Clifton, NJ). TLC plates were developed twice in butanol:propanol:water (3:14:4). Fructan was detected by urea-phosphoric acid stain (Wise et al. (1955) *Anal. Chem.* 27:33-36). Analysis 35 demonstrated that control seeds (untransformed) did not contain fructan. TLC plates also demonstrated that seeds expressing the SST gene accumulated fructan with a degree of polymerization (DP) of 3 (Figure 3).

EXAMPLE 2Chimeric Construct for Expression of the
Jerusalem Artichoke FFT Gene in Transgenic *Zea mays* L.

A construct designed for tissue specific expression of the Jerusalem Artichoke FFT gene in maize endosperm was assembled by replacing the Cauliflower Mosaic Virus (CaMV) 35S promoter in the plasmid pSST405 (Van Tunen et al., WO 96/21023) with a maize endosperm-specific 10 kD zein, seed storage gene promoter (Kirihera et al. (1988) *Gene* 71:359-370). The complete FFT coding sequence contained in pSST405 (Van Tunen et al., WO 96/21023) 5 was isolated by digesting with the restriction endonuclease enzymes NcoI and BamHI. The isolated sequence was added to the plasmid pCyt-SacB (Caimi et al. (1996) *Plant Physiol.* 110:355-363) containing a 10 kD zein promoter and 3' 10 termination region. pCyt-SacB was digested with NcoI and BamHI to remove the SacB region. The maize endosperm expression cassette, containing the 10 Kd 15 promoter, FFT coding sequence (including the native secretory and vacuole targeting signals) and the 10 kD 3' end, designated 10 kD-FFT (Figure 1), was isolated by digesting with SmaI and SalI, then ligated into the plasmid KS17. The final vector was designated 10 kD-FFT-17 was used directly for transformation into corn by particle co-bombardment with the plasmid 10 kD-SST-17, and 20 pDetric described in Example 1. Transformation, regeneration and growth to mature plants was by the methods described in Example 1.

Analysis of Transgenic Plants Expressing the SST and FFT Genes

Detection of the FFT gene in transgenic plants co-bombarded with SST and FFT genes was accomplished by PCR analysis, using oligonucleotide primers 25 specific for the FFT coding sequence:

FFT-1: 5' -CCCTGAAACCTTACAGACCTTGAAACATGAACCCCA-3' (SEQ ID NO: 3)

FFT-2: 5' -GGCGGAAATCTTGAGAGATGCTTCACACTCGTACC-3' (SEQ ID NO: 4)

30 Confirming the presence of the FFT gene in transgenic tissue and estimating the copy number was done by Southern analysis, using the complete 2.0 Kb FFT coding sequence. Southern analysis demonstrated the presence of multiple intact and rearranged copies of the FFT gene in transgenic lines (Figure 4).

Carbohydrate Analysis of Maize Lines Containing the SST and FFT Genes

Transgenic seeds expressing the SST and FFT genes were harvested at 25-35 DPP. Isolation and detection of fructan was described in Example 1. Fructan was detected by urea-phosphoric acid stain (Wise et al. (1955) *Anal. Chem.* 27:33-36). Analysis demonstrated that control seeds did not contain

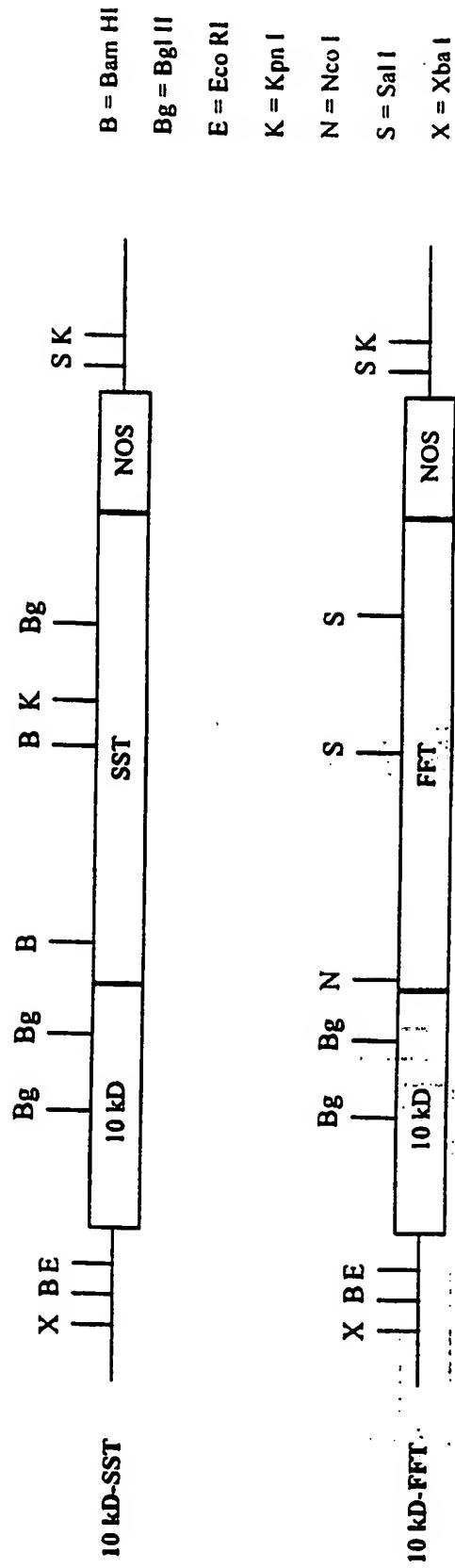
fructan. TLC plates also demonstrated that seeds expressing both the SST and FFT genes accumulated fructan with a DP much larger than in lines containing the SST gene alone (Figure 5). The results demonstrate that the FFT gene acts as a chain elongation factor, synthesizing fructan with a DP of at least 20 (the limit of 5 detection by TLC) in transgenic seeds.

CLAIMS

What is claimed:

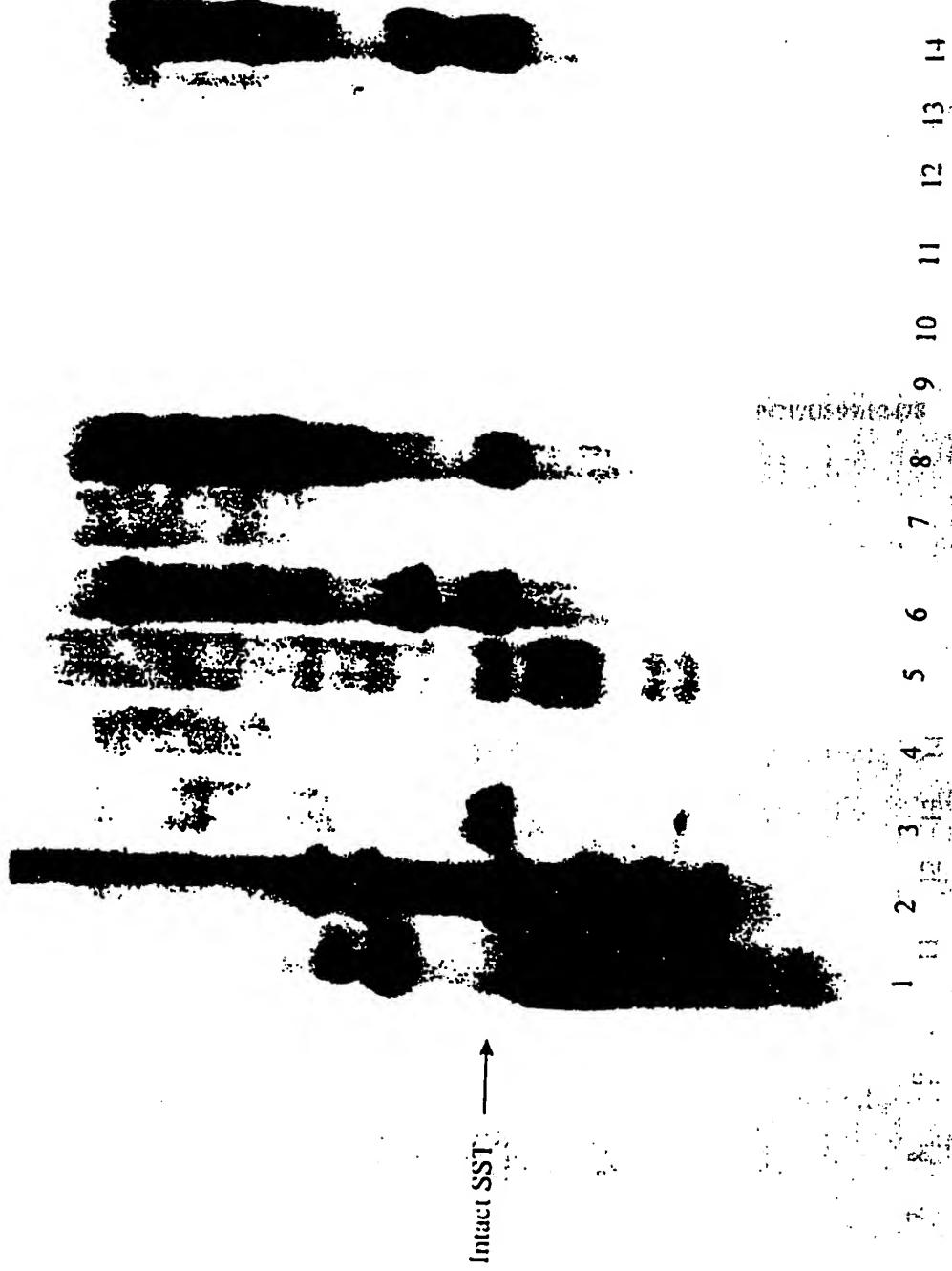
1. A method of increasing the level of fructan that accumulates in the cells of a transgenic monocot plant comprising:
 - 5 a) preparing at least one chimeric gene comprising a plant fructosyltransferase gene operably linked to suitable regulatory sequences that function in monocot cells;
 - b) transforming a monocot cell with the at least one chimeric gene; and
 - 10 c) regenerating a transgenic monocot plant from the transformed monocot cellwherein the level of fructan that accumulates in the cells of the transgenic monocot plant is increased when compared to the level of fructan that accumulates in the cells of a monocot plant comprised of cells that do not contain 15 the at least one chimeric gene.
2. The method of Claim 1 wherein the plant fructosyltransferase gene encodes a sucrose-sucrose-fructosyltransferase.
3. The method of Claim 2 wherein the monocot cell is transformed with a second chimeric gene comprising a plant fructose-fructose-fructosyltransferase 20 gene operably linked to suitable regulatory sequences that function in monocot cells, and wherein the fructan that accumulates in the cells of the transgenic monocot plant has a degree of polymerization of at least 20.
4. The method of Claim 1 wherein the suitable regulatory sequences include a tissue specific promoter.
- 25 5. The method of Claim 4 wherein the tissue specific promoter directs expression of the operably linked fructosyltransferase gene in the seeds of the transgenic monocot plant.
6. The method of Claim 1 wherein the transgenic monocot plant is *Zea mays*.
- 30 7. A chimeric gene comprising a plant fructosyltransferase gene operably linked to suitable regulatory sequences that function in monocot cells.
8. The chimeric gene of Claim 7 wherein the plant fructosyltransferase gene is a member selected from the group consisting of a sucrose-sucrose-fructosyltransferase gene and a fructose-fructose-fructosyltransferase gene.
- 35 9. The transgenic monocot plant produced by the method of Claim 1.
10. Seeds from the plant of Claim 9 wherein the seeds comprise chimeric genes comprising a plant fructosyltransferase gene operably linked to suitable regulatory sequences that function in monocot cells.

Figure 1



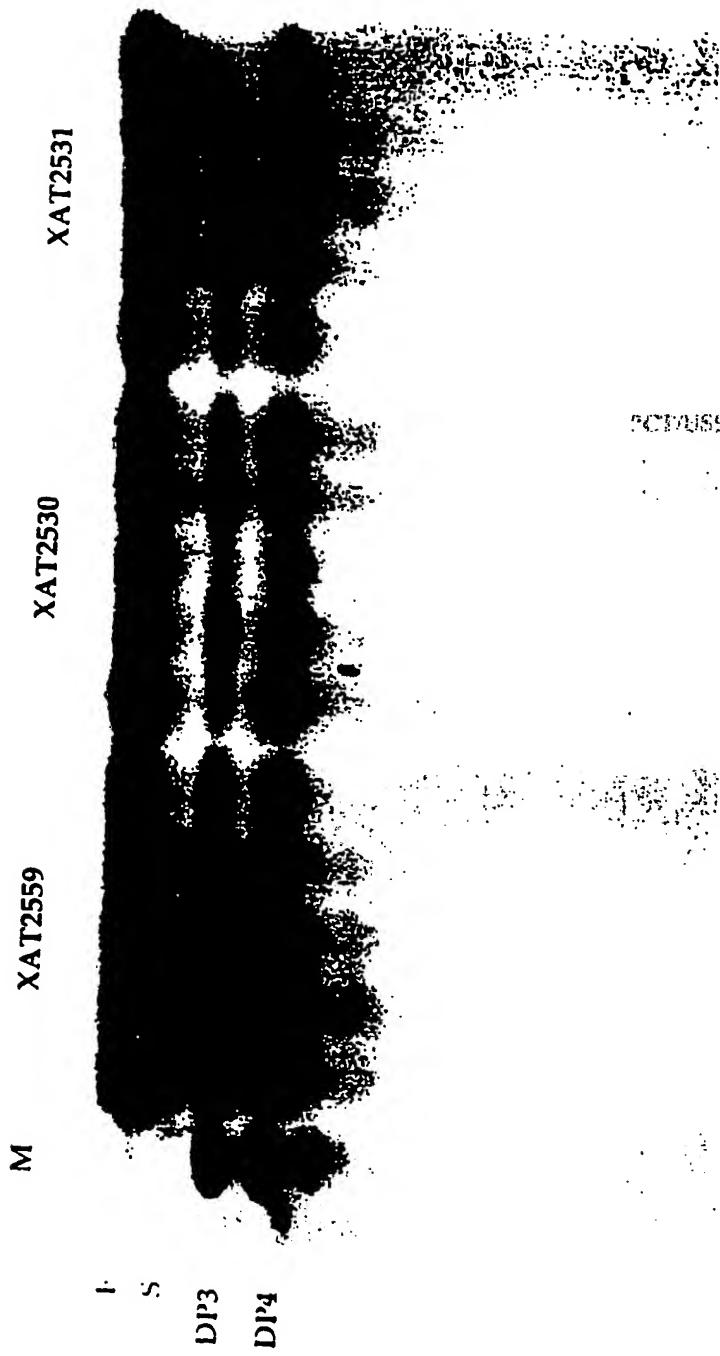
2/5

Figure 2



3/5

Figure 3



PCT/US99/02478

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Impact 1:1

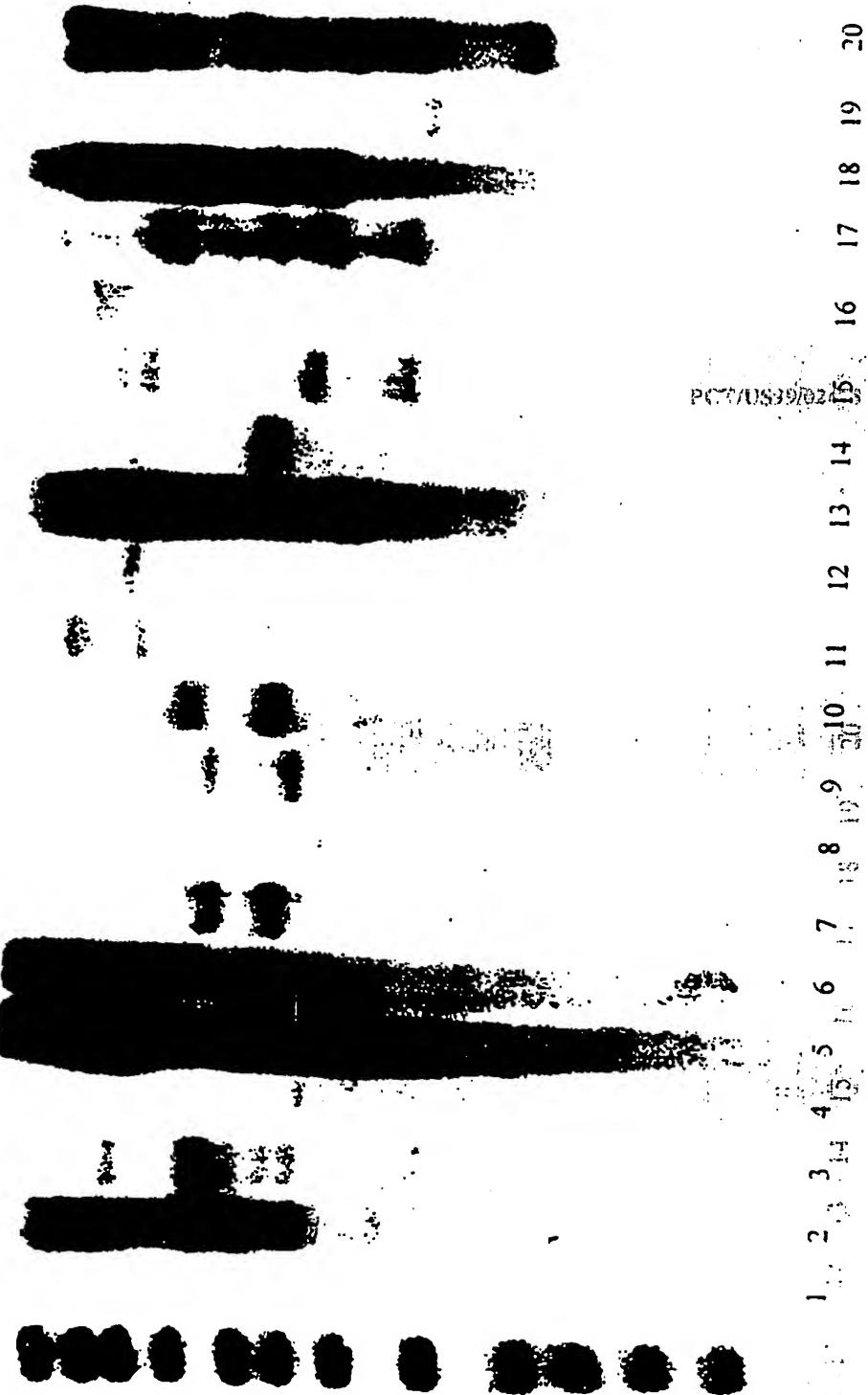
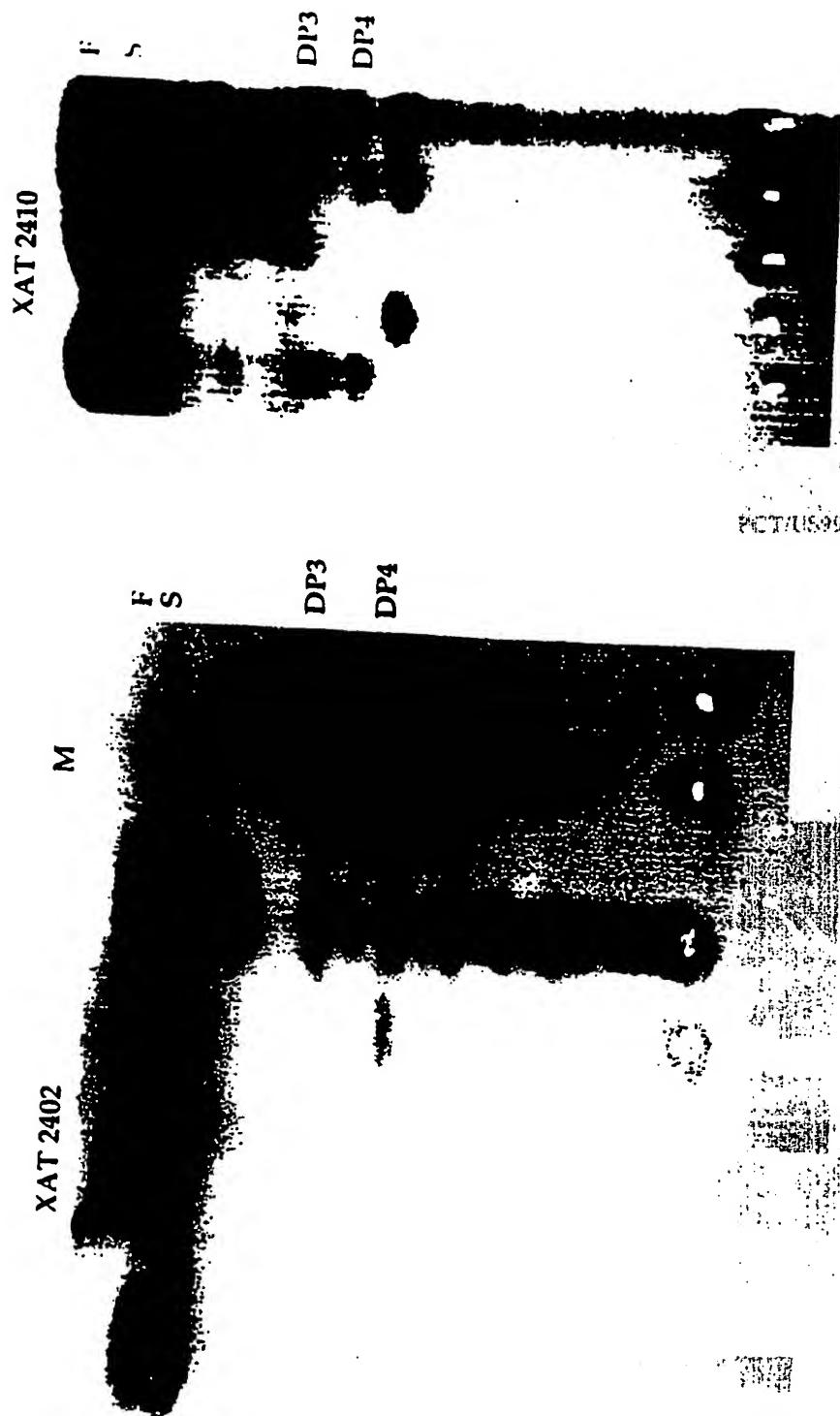


Figure 4

5/5

Figure 5



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
- (B) STREET: 1007 MARKET STREET
- (C) CITY: WILMINGTON
- (D) STATE: DELAWARE
- (E) COUNTRY: USA
- (F) ZIP: 19898
- (G) TELEPHONE: 302-992-4926
- (H) TELEFAX: 302-773-0164
- (I) TELEX: 6717325

(ii) TITLE OF INVENTION: TRANSGENIC CROPS ACCUMULATING
FRUCTOSE POLYMERS AND METHODS
FOR THEIR PRODUCTION

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
- (B) COMPUTER: IBM PC COMPATIBLE
- (C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
- (D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 60/077,727
- (B) FILING DATE: MARCH 12, 1998
- (C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: MAJARIAN, WILLIAM R.
- (B) REGISTRATION NUMBER: 41,173
- (C) REFERENCE/DOCKET NUMBER: BB-1082-A

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: SST-1
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAATCCTT TCATCCACCA CGACCACCCC TCTC

34

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: SST-2
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCAAGGAAGA GGGAAAGGAT TGAGTTCTGC TTCCCC

36

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: FFT-1
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCCCTGAACC CTTTACAGAC CTTGAACATG AACCCCA

37

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (vii) IMMEDIATE SOURCE:
 - (B) CLONE: FFT-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGCGGAAAT CTTGAGAGAT GCTTTCACAC TCGTACC

37

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 99/02478

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N9/10 C12N5/10 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 13389 A (DU PONT ;CAIMI PERRY GERARD (US); HERSHY HOWARD PAUL (US); KERR P) 18 May 1995 (1995-05-18) examples 6-8 ---	1-10 02478
Y	WO 96 01904 A (STICHTING SCHEIKUNDIG ONDERZOE ;SMEEKENS JOSEPHUS CHRISTIANUS (NL)) 25 January 1996 (1996-01-25) cited in the application see examples claims 5-8 ---	1-10
A	WO 96 21023 A (CT VOOR PLANTENVEREDELINGS EN ;TUNEN ARJEN JOHANNES VAN (NL); MEER) 11 July 1996 (1996-07-11) cited in the application the whole document ---	1-10 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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Date of the actual completion of the international search 28 July 1999	Date of mailing of the international search report 04/08/1999
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INTERNATIONAL SEARCH REPORT

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